Advantages and limitations of commonly used methods to assay the molecular permeability of gap junctional intercellular communication

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The role of gap junctional intercellular communication (GJIC) in regulation of normal growth and differentiation is becoming increasingly recognized as a major cellular function. GJIC consists of intercellular exchange of low molecular weight molecules, and is the only means for direct contact between cytoplasms of adjacent animal cells. Disturbances of GJIC have been associated with many pathological conditions, such as carcinogenesis or hereditary illness. Reliable and accurate methods for the determination of GJIC are therefore important in cell biology studies. There are several methods used successfully in numerous laboratories to measure GJIC both in vitro and in vivo. This review comments on techniques currently used to study cell-to-cell communication, either by measuring dye transfer, as in methods like microinjection, scrape loading, gap-fluorescence recovery after photobleaching (gap-FRAP), the preloading assay, and local activation of a molecular fluorescent probe (LAMP), or by measuring electrical conductance and metabolic cooperation. As we will discuss in this review, these techniques are not equivalent but instead provide complementary information. We will focus on their main advantages and limitations. Although biological applications guide the choice of techniques we describe, we also review points that must be taken into consideration before using a methodology, such as the number of cells to analyze.

INTRODUCTION

Most cells in multicellular organisms perform gap junctional intercellular communication (GJIC). This is the well-known form for direct contact between cytoplasms of adjacent animal cells. The juxtaposition of two half-channels, called connexons, constitutes this junction (Figure 1). Each connexon is inserted into the cytoplasmic membrane of two neighboring cells. It is made of six protein subunits called connexins (Cx) and can be homomeric or heteromeric (1). Connexons are absent from spermatocytes, erythrocytes, thrombocytes, adult skeletal muscle, and some neuronal subpopulations (2). The Cx subunit is a four-transmembrane spanning protein, harboring two extracellular loops, a cytoplasmic loop, and a cytoplasmatic N- as well as C-terminal region (Figure 1). The Cx family in humans is composed of 21 types of proteins classified according to their molecular weight. One cell type can express more than one Cx isoform (3). The flux of molecules through these channels includes the passive diffusion of small (<1 kDa) and hydrophilic molecules, such as metabolites (e.g., ATP), nutrients (e.g., glucose), and second messengers (e.g., triphosphate inositol, ionic coupling, and Ca^{2+}) (4). GJIC was for a long time regarded as a relatively passive way of intercellular signaling through cell-to-cell tunnels; however, it was finally recognized that the degree of intercellular coupling is finely regulated in three main aspects: (*i*) the number of channels present in the membrane, (*ii*) their functional state, and (*iii*) their permeability (5). These channels are critical to several physiological roles, such as impulse propagation in the heart and neurons (6), response of tissues to hormones (7), regulation of embryonic development (8), homeostasis (9), and regulation of cellular proliferation (10).

Gap junction channels can be regulated by several agents, including voltage, phosphorylation, intracellular calcium and pH, adhesion proteins, extracellular matrix, and hormones (11).

Studies of cell-to-cell communication are carried out either by measuring dye transfer using techniques such as microinjection, scrape loading, electroporation, gap-FRAP (fluorescence recovery after photobleaching), preloading assays, local activation of molecular fluorescent probe (LAMP), or by measuring electrical conductance and metabolic cooperation.

In this paper, we describe fluorescent dye transfer techniques and other methods commonly used to study GJIC capacity in vitro and in vivo. Currently, all of these techniques are widely used to understand the physiology of gap junctions; for instance, to study the modifications to their normal function by chemicals, carcinogenesis, cell growth, and embryogenes, or to study the permeability and ion selectivity of Cx isoforms. Knowledge of reliable and accurate methods for the determination of GJIC functionality and permeability is therefore important in studies of cell biology.

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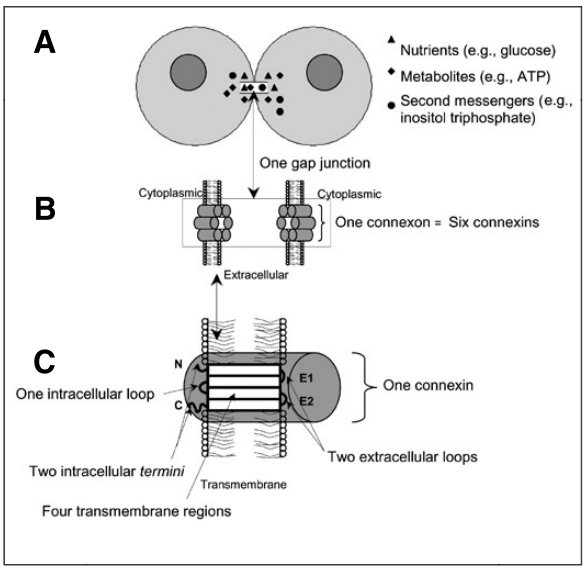

Figure 1. Basic intercellular communication topology and gap junction structure. (A) Two cells are coupled by a variable number of gap junction channels that allow the passage of small molecules as described in the introduction. (B) Six protein subunits called connexins oligomerize into a hexameric structure called the connexon. Two connexons constitute one gap junction. (C) Transmembrane topology of a connexin protein, including four transmembrane regions, two extracellular loops, an intracellular loop, and two intracellular *termini*.

DYE TRANSFER

Most of the methods available for investigating intercellular communication mediated by Cx channels are dependent on the introduction into living cells of nontoxic dyes that are traced in their eventual intercellular movements. Molecules suitable for such experiments should be small enough to cross gap junction channels, whose cut-off size is around 900 Da, and should not leak across a normal nonjunctional membrane. Most Cx channels are permeable to several tracers and until now, no dye has been shown to permeate only one type of gap junction channel, that is, to be specific for any Cx species. However, it is also clear that different Cx form channels with distinct permeabilities and that these specific properties may allow for subtle discrimination between molecules that differ only in size and/or electrical charge (12).

Microinjection

Principle. Microinjection of membrane-impermeable, nontoxic tracers has been the first technique used to identify and map the communication network of a wide variety of cell systems. Indeed, the existence of functional cell-to-cell channels can be verified by intercellular transfer of tracers, which requires intracellular injection of membrane-impermeable but gap junction– permeable dye (13).

The first cell-to-cell spread of a fluorescent diffusion tracer introduced into one cell by microinjection was demonstrated by Kanno and Loewenstein in 1964. A fluorescent dye within the micropipette (with a tip diameter about 0.2 μm) is injected intracellularly by an electrical or pressure pulse (Figure 2A). This technique introduces macromolecules into cells by a transient perturbation of the cell membrane, which does not affect cell viability (Table 1). If the adjacent cells are coupled by gap junctions, the dye will diffuse and label them. Fluorescein was the first fluorescent dye extensively used to characterize direct intercellular coupling (14). However, nonjunctional membrane was found to be permeable to this dye (15) and thus fluorescein was gradually replaced by Lucifer yellow (LY) (16). The choice of the most adequate tracer is dependent on several factors, including the scope of the experiment and the conditions under which the study is conducted.

Advantages. Because it enables a selective loading of dyes, microinjection permits the correlation of morphological and functional data from individual cells. Furthermore, because the duration of the tracer injection can be accurately controlled, the technique is also instrumental in kinetic studies aimed at evaluating the rate of transfer from one cell to another. Moreover, the microinjection method has the advantage that the level of cell communication is expressed as number of dye-coupled cells, which gives a basis for direct comparison of cell communication in different cell types (17). This approach is still widely used to study cell coupling in cultures and is the main technique available to extend such studies to intact tissues.

Limitations. The main limitation of microinjection is that it requires specialized equipment and technical skill to prevent cell damage, or at least to detect damage during injection of the dye. Also, performing intracellular microinjection requires some dexterity, and the amount of microinjected tracer is difficult to standardize (18). As is often mentioned, results obtained by this method vary with the investigator's experience in impaling cells without causing too much damage and also in discriminating stained from unstained (autofluorescent) cells (19). Cell impalement causes sudden and dramatic changes in intracellular homeostasis, including changes in membrane potential or intercellular ionic concentration, which in turn can alter GJIC (20,21).

A simulation, for instance a mechanical stretch, cannot be applied simultaneously with microinjection. Microinjection is thus unsuitable for detecting effects that are either very rapid or require a continuous application of the stimulus (20).

The technique is not convenient when a large number of cells need to be assessed simultaneously for intercellular communication. In practice, only a few cells may be microinjected, usually not at the same time (13). Quantitation of dye transfer is mainly based on visual observation and manual counting of dye-coupled cells. Manual cell counting is however both time-consuming and inaccurate. Cell counting has inherent problems because it is dependent on cell density and cell morphology. Also, the more cells the dye passes to, the more dilute it gets. Therefore, it is important to make sure that inter-experimental conditions are identical. Because of these considerations, some experimenters look only at dye passage to first-generation cells or nearest neighbors. When a dye injection assay is used, it is difficult to determine subtle differences in gap junction coupling as a consequence of treatment (22); thus, other methods are needed to evaluate more subtle effects.

Significant studies and applications. Dye transfer is a relatively crude but widely used method for demonstrating

intercellular communication through gap junctions in monolayer cells (23,24) and tissue in various biological circumstances (Table 2).

A selection of different fluorescent dyes with divergent molecular masses and charges is available (Table 1) and therefore, this technique appears appropriate to study junctional permeabilities and exclusion limits of channels (25–27). Several diffusion models have been developed in monolayer cultured cells or in *Xenopus* oocytes (28–30). Different Cx form channels with distinct permeabilities, allowing a subtle discrimination between molecules that are only slightly different in size and/or electrical charge. Hence, some Cx channels may allow for the passage of some, but not all, available tracers (31). Dyes that bind strongly to DNA, such as propidium iodide, ethidium bromide, and DAPI (4′,6-diamidino-2-phenylindole) dihydrochloride, are limited in their utility as diffusible tracers (32).

The liver is an organ frequently used to study GJIC by the dye transfer assay (33). Krutovskikh et al. developed a

Table 1. General Features of Gap Junction–permeable Dyes Used for Intercellular Communication Assessment

Name	Abbreviations	Absorption Wavelength (nm)	Emission (nm)	Net Charge[a]	Molecular Weight (Da)	Methods	Ref.
hydroxycoumarin carboxylic acid	HCCA	386	448	- 6	206	MI	(123)
calcein blue	-	360	449	0	321	MI	(123)
N-2[aminoethyl]-biotinamide hydrochloride	Neurobiotin	Nonfluorescent		+1	287[b]	SL, IL	(47,124)
4′,6-diamidino-2-phenylindole dihydrochloride	DAPI dihydrochloride	358	461	+1	279[b]	MI	(32)
ε-biotinoyl-L-lysine	Biocytin	Nonfluorescent		0	372.5	SL, MI	(125,126)
5-(and 6)- carboxyfluorescein diacetate	CFDA	Nonfluorescent		0	376 (CF)	Gap-FRAP	(60)
carboxyfluorescein	CF	492	517	-2		MI	(127)
ethidium iodide(bromide)	EthBr	518	605	+1	314[b]	SL, MI	(32)
2′,7′- dichlorofluorescein	diCl-F	504	529	-1	401	MI	(128)
1-(2-nitrophenyl)ethyl -hydroxy-6-chloro-coumarin 3-carboxamide acetoxymethyl ester	NPE-HCCC2/AM	Nonfluorescent		ND	450 (HCCC2)	LAMP	(40)
7 hydroxy-6-chloro-coumarin 3-carboxamide	HCCC2	408	448				
dicarboxy-dichlorofluorescein diacetate	CDCFDA	Nonfluorescent		-2	445 (CDCF)	Gap-FRAP, PA	(129,130)
dicarboxy-dichlorofluorescein	CDCF	504	529	ND			
Lucifer yellow CH	LY	428	536	-2	443[b]	SL, MI	(131)
2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester	BCECF-AM	Nonfluorescent		0	520 (BCECF)	FC, Gap-FRAP	(132)
2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein	BCECF	503	528	-4,-5			
alexa fluor 488	-	495	519	-1	570.5	MI	(35)
cascade blue	-	400	420	ND	607	MI	(133)
calcein acetoxymethyl ester	Calcein-AM	Nonfluorescent		0	622 (calcein)	Gap-FRAP, PA, FC	(58)
calcein	-	494	517	-4		MI	(134)
propidium iodide	PI	535	617	+2	414[b]	MI	(32)
lissamine rhodamine B200	LB200	575	595	0	559	MI	(135)

[a]At neutral pH.
[b]Molecular mass of permeating ion analyzed (without counter ion) from Elfang et al. (32).
FC, flow cytometry; FRAP, fluorescence recovery after photobleaching; IL, incision loading; LAMP, local activation of molecular fluorescent probe; MI, microinjection; PA, parachute assay; SL, scrape loading; ND, not determined.

simple method to measure GJIC in liver slices freshly removed from the rat to study sequential change of GJIC during chemical hepatocarcinogenesis (34). In brief, injections of LY were performed with a handmade glass needle into a fresh thin slice of rat liver. Injected slices were quickly washed in phosphate-buffered saline, embedded in gelatin, frozen, and cut into cryosections. Each spot of LY dye spread on serial sections was photographed under a fluorescence microscope. With this approach, it could not be strictly ruled out that the slices varied in thickness and, therefore, hepatocytes in the center of the sections could be damaged due to a restricted supply of oxygen or nutrients (35).

In the field of embryogenesis, Levin and colleagues suggested that endogenous dorsoventral differences in GJIC within the early embryo are needed to consistently orient left-right asymmetry (36). The authors used 8- or 16-cell *Xenopus* embryos fixed in paraformaldehyde in which LY was microinjected into one cell.

Scrape Loading

Scrape loading is a simple alternative to microinjection for introduction into cultured cells of tracers that cannot cross the cell membrane.

Principle. In this approach, monolayers of adherent cells are scraped or scratched in the presence of a gap junction permeable tracer, which becomes incorporated by cells along the scrape, presumably as a result of some mechanical perturbation of the membrane (Figure 2B). As normal membrane permeability is re-established, the tracer becomes trapped within the cytoplasm and, with time, may move from the loaded cells into adjacent ones connected by functional Cx channels (37). The distance at which the fluorescent dye diffuses during a certain period away from the scrape line is indicative of GJIC level (38). The optimal incubation time may vary between different cell types, depending on the level of communication and attachment properties of the cells (39). For a set experiment duration, different rates of dye diffusion through homotypic channels between different cell types is correlated to the number of gap junction channels (19). Other fluorescent dyes, such as rhodamine-dextran (RhD) or dialkylcarbocyanine (Di-I), which are larger than the gap junction channel limit, are used as a control to label the initially loaded cells and to show that dye transfer from cell to cell is not by other means, such as cytoplasmic membrane fusions or cytoplasmic bridge formation, or due to leaky membranes, which can occur under cytotoxic conditions (38).

Advantages. The major advantages of the scrape-loading approach are that (*i*) it does not necessitate most of the special equipment and skills that are needed for microinjection, and (*ii*) it is possible to conduct a rapid and simultaneous assessment of junctional communication in a large number of cells.

It is the fastest and simplest technique to measure GJIC because a straight scrape made by a scalpel (or a needle) on monolayer cultured cells efficiently allows fluorescent dye to enter through this wound. It is therefore particularly useful when a large screen of multiple conditions is required or when different regions of a cell monolayer have to be compared within the same culture dish. Scrape loading can measure dye transfer in multiple cells almost simultaneously, and it is the only visual method available to assess the overall GJIC of a population of cells (38). The scrape-loading technique can be effectively used as a tool to determine the qualitative presence or absence of GJIC.

Limitations. The approach is inappropriate to investigate small cells or cell assemblies (such as pairs), as well as low-density cultures. It is complicated when the extent of junctional coupling is small or when selected cells have to be individually screened for coupling. It is not well suited to three-dimensional (3-D) systems because this invasive method may introduce uncertainties in quantifying dye transfer rates due to variations in cell-staining intensity after scrape loading (40).

The scrape-loading technique is not useful for Cx channels that exclude molecules such as LY (38). For example, Cx45 channels cannot be assessed using LY (41). If the Cx channels of a particular cell are unknown, then the scrape-loading technique using LY as the transfer dye does not necessarily represent an accurate portrayal of the GJIC "competency" of that cell population. Dye coupling should be interpreted with caution because two different tracers may not be detected equivalently (42). For channels that exclude LY, smaller dyes such as biotin conjugates have been used (Table 1).

The GJIC levels of some cell types of irregular shape are not easily quantified using the scrape-loading assay. For example, gap junction function of neuronal cells with extensive processes or long spindly fibroblasts cannot be easily quantified because the distance dye travels is not easily "trackable" (38). The problem of nonuniform labeling with LY or variation in the intensity after scrape loading has been reported previously (43).

As a result, techniques of microinjection and scrape loading are widely used to qualitatively demonstrate intercellular communication, although the use of image analysis in combination with scrape loading was reported to produce quantitative information and to reduce considerably the statistical variation in the data (39).

Significant studies and applications. Scrape loading has been used to assess the GJIC status of many cell types in various biological circumstances (Table 2). Sai et al. reported an in vivo GJIC assay, the incision loading/dye transfer method (IL/DT) using LY and RhD (44). RhD, which does not pass through the channels, is used

to identify the incision sites or damaged cells. With this double dye transfer, authors examined whether drinking green tea might prevent GJIC inhibition by a tumorigenic compound, pentachlorophenol, in the liver of mice. Romualdi et al. described an improvement to this dye transfer method in which precision-cut slices (250 µm cross-section) from mouse liver were used to quantify GJIC after iontophoretic injection of the fluorescent dye Alexa Fluor 488 directly into hepatocytes (35). Dye injection and spreading were monitored in real time and quantified by fluorescence microscopy. The integrity and viability of the cells were controlled by monitoring the membrane potential.

The IL/DT method illustrates the potential benefit of detecting and predicting effective doses for tumorigenesis. This method has many advantages for studying tumor-promoting activity in vivo, because even though the Cx expression level recovered at an early stage after treatment with the tested compound, a low level of GJIC persisted for a longer time, indicating that an analysis of the expression level of Cx, such as for protein, mRNA, or its localization, does not always reflect gap junction function in vivo. With respect to devising a much simpler and quicker procedure compared with the microinjection method, IL/DT may be useful for rapidly screening tumorigenic compounds for setting doses for studies of carcinogenesis (44).

The analysis of GJIC by microinjection or scrape loading can also be used with sections of other tissues, such as skin (45), testis (46), and central nervous system tissue (47). An exhaustive protocol was described by Meda's group in primary tissues (13).

Electroporation

Principle. Raptis et al. proposed a new way to introduce nonpermeable fluorescent dye into adherent cells on a partly conductive slide, called electroporation. (48). Cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium-tin oxide. An electric pulse that opens transient pores on the plasma membrane is applied in the presence of the fluorescent dye LY, causing the dye's penetration into the cells that are growing on the conductive part of the slide. Cells growing on the nonconductive slide are not permeabilized as it does not receive any current (Figure 2C). Migration of the dye through gap junctions to nonelectroporated cells is then microscopically observed under fluorescence illumination (49). To quantitate gap junctional coupling, the number of cells into which dye transferred through gap junctions per electroporated border cells was calculated by dividing the total number of labeled cells on the nonconductive slide by the number of cells growing at the border with the conductive coating (Figure 2C) (50).

Advantages. Lucifer yellow can enter simultaneously into large numbers of cells with minimal disturbance to cellular metabolism (45). This technique can be applied to a large variety of adherent cell types (50). According to De Vuyst et al., electroporation loading and dye transfer (ELDT) is a robust technique to investigate gap junctional coupling that combines minimal cell damage with accurate probing of the degree of cell-to-cell communication. It is a satisfactory method for loading a narrow longitudinal strip of cells for subsequent studies on the temporal and spatial spread of fluorescent dyes via gap junctions (51).

Limitations. This technique is not recommended to examine cell types that do not adhere well to their solid support. If cells are grown to high confluence, they may detach due to the turbulence and suction forces created as the top electrode is removed after electroporation and GJIC study becomes impracticable. GJIC evaluation is also difficult when a small part of the cell layer is separated during removal of the electrode because the dye will diffuse under the cell sheet (52). An accurate determination of optimal voltage is important to not induce damage of the electroporated cells at the border with the nonconductive area (50).

Significant studies and applications. Recently, Raptis et al. have described a slide configuration that eliminates the upper electrode, which can induce cell detachment when it is removed. Cells are grown on two co-planar electrodes, an approach that is valuable for the examination of cells that do not adhere closely to their solid support (52). To investigate the extent of dye spread between C6 glioma cells wild-type and transfected with Cx43 and Cx32 coupled by gap junctions, De Vuyst et al. proposed bipolar-pulsed high frequency electroporation by a small two-wire electrode positioned close to the cells (51).

Gap-FRAP (Fluorescence Recovery after Photobleaching)

Principle. The irreversible photochemical bleaching reaction of fluorescent molecules exposed to a light beam of sufficient intensity and of wavelength within the absorption spectrum of the fluorochrome forms the basis of the FRAP (fluorescence recovery after photobleaching) techniques. FRAP experiments started in the 1970s using lipophilic or hydrophilic fluorophores such as fluorescein coupled to protein and lipid (53,54). The FRAP method has been devised for measuring the two-dimensional diffusion of fluorescently labeled macromolecules in membranes, cytoplasm, and organelles. Fluorescent molecules in a small region of the cell are irreversibly photobleached using a high-powered laser beam, and subsequent movement of surrounding nonbleached fluorescent molecules into the photobleached area is recorded at low laser power (55). FRAP data requires that the bleach event is much shorter than the recovery time and preferably as short as possible (56). Fluorescence recovery occurs in the bleached area. Corresponding diffusion constants or flow velocities are determined by analysis of the spatial and temporal kinetics of fluorescence recovery. The gap-FRAP technique enlarges the FRAP technique to study the functionality of gap junctional intercellular communication channels (57). In the gap-FRAP technique, the size and the chemical and biophysical properties of the fluorescent molecules define the communication capacity, allowing quantification of this functionality even if the tracers have no metabolic role (57). A fluorescent diffusion tracer is first introduced at uniform concentration into all the cells in a tissue culture dish, and a concentration gradient is created by photobleaching the fluorescence in one cell. If the bleached cell communicates with neighbor cells by means of functional gap junction channels, resolution of this concentration gradient is detected by monitoring the fluorescence signal, using low-intensity excitation (Figure 2D) (18). Typically, cells are loaded with

lipophilic tracers such as 5(6)-carboxyfluorescein diacetate (CFDA) or calcein acetoxymethyl ester (AM) (Table 1), and dyes are retained inside the cells due to the hydrolysis by cytoplasmic esterases into carboxyfluorescein (CF) or calcein, respectively. According to their low molecular weights (376 Da and 622 Da, respectively, Table 1), they have been shown to permeate gap junction channels (58–60). After the loading process, the cell culture slides are washed several times to remove the fluorochrome-ester and to prevent further dye loading during subsequent measurements. This is followed by a light irradiation, leading to dye photobleaching in the targeted cell. If the tracer is hydrophilic and its molecular weight lower than 1 kDa, recovery of fluorescence in the bleached area is the result of an influx of unbleached dye from neighboring cells via gap junctions. The degree of fluorescence recovery is directly proportional to the degree of functional gap junction coupling. Assuming that the fluorescence signal measured under constant experimental conditions is proportional to dye concentrations, the equation can be written as:

$$F(t) = F_o + (F_\infty - F_o)(1 - e^{-t/\tau}) \ , \qquad \text{[Eq. 1]}$$

where $F(t)$ is the normalized fluorescence intensity at time t after photobleaching; F_∞ is the asymptotic value to which the fluorescence intensity tends; F_o is the theoretical fluorescence intensity estimated at t = 0 s after photobleaching, which is close to its experimental value; and τ is the time constant.

Advantages. To follow the functionality of GJIC, the gap-FRAP technique shows advantages compared with other strategies, being noninvasive, easier, and faster than approaches such as dye transfer by microinjection. Moreover, as it was demonstrated by the cell-to-cell spread of a fluorescent diffusion tracer introduced into a cell by microinjection or scrape loading, the permeability of the gap junctions is not restricted to the small intracellular electrolytes that carry the junctional currents (14,37). Quantitative data on the permeability of the gap junctions for larger solutes, which include second messengers and other signaling molecules, contribute to the understanding of their function. Microinjection and scrape-loading methods provide information on the expression of functional gap junctions and on the size of the permeating molecules, but permeability coefficients have usually not been obtained with these techniques (18).

The gap-FRAP approach enables users to quantify and compare GJIC capacity between characterized cell types. Kinetic curves can be established to precisely measure the functionality of GJIC. Gap-FRAP is useful to study individual cells within a population. With the gap-FRAP method, the initial and boundary conditions of the diffusion system can usually be reduced, with a precision sufficient for practical purposes, to those of a simple system of two compartments separated by a permeable membrane. The first compartment comprises the cell or the set of cells connected by gap junctions to the

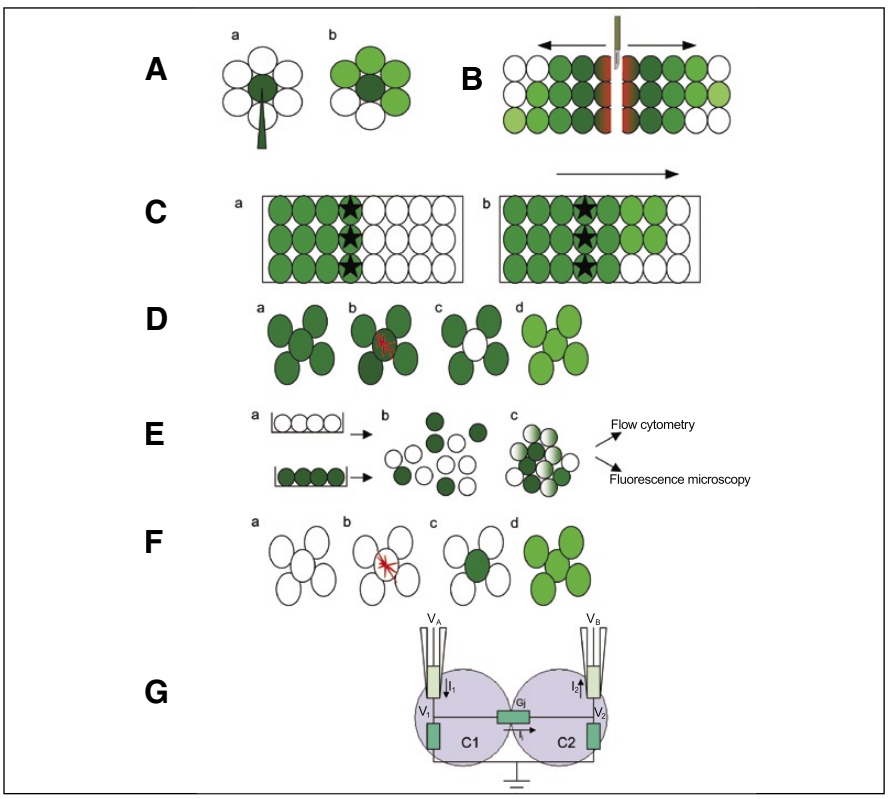

Figure 2. Methods to study gap junctional intercellular communication (GJIC). (A) Microinjection: (a) injection of fluorescent membrane–impermeable but gap junction–permeable dye (green) is performed in the targeted cell; (b) if the adjacent cells are coupled by gap junctions, dye will diffuse and label them, otherwise the adjacent cells remain unlabeled. (B) Scrape loading: Cells along the scraped line are loaded with fluorescent dye (green) and a nonpermeable fluorescent dye (red). The tracer diffuses to the neighboring cells in a few minutes through gap junctions. The distance the fluorescent dye travels within a designated period away from the scrape line is indicative of GJIC. (C) Electroporation: (a) an electric pulse is applied, causing the dye's penetration (green) into the cells growing on the conductive part of the slide; (b) migration of the dye through gap junctions to nonelectroporated cells is observed. Border cells on conductive slide are represented by black stars. (D) Gap-FRAP (fluorescence recovery after photobleaching): (a) cells after dye exposure; (b) cells during photobleaching of the targeted cell; (c) cells just after the photobleaching time; (d) end of fluorescence recoveries through gap junctions in the targeted cell. (E) Preloading assay: (a) green cells are "donor" cells, preloaded with gap junction–permeable dye and white cells are "acceptor" cells, unloaded with gap junction–impermeable dye; (b) "donor" and "acceptor" cells plated together to form confluent monolayer cells; (c) after a short time the fluorescent dye will pass from the "donor" cells to "acceptor" cells. This transfer characterizes efficient gap junction channels and is analyzed either by flow cytometry or fluorescence microscopy. (F) LAMP (local activation of molecular fluorescent probe): (a) cells after dye exposure; (b) cells during photoactivation of the targeted cell; (c) cells just after the illuminating time; (d) fluorescence diffusion through gap junctions to neighboring cells. (G) Dual whole-cell patch clamp applied on equivalent circuit for a pair of cells C1 and C2. Voltage is imposed by source V_A and V_B into cells through electrodes V_1 and V_2, respectively. When a voltage command step is applied to C1, an incremental current is required to maintain the voltage of C1. Electric current (I_1) flows from the C1 to the C2 and to earth, through junctional and nonjunctional pathways, respectively. Since C2 is voltage-clamped, the patch clamp amplifier produces the electric current (I_2) in order to maintain a constant cell potential. I_2 is equal in magnitude and opposite sign to I_j (junctional current). The junctional conductance (G_j) can be calculated by simply dividing the junctional current by the voltage step applied to C1 (109,122).

photobleached cell, which constitutes the second compartment. Furthermore, with appropriate caution, the risk of cell injury, which in microinjection or scrape-loading experiments requires constant attention and may be difficult to recognize, is usually negligible with the gap-FRAP method. A further advantage of the gap-FRAP technique is the possibility to obtain paired data in control and test conditions and to perform relatively long-term observation on the same cells (18).

Limitations. The gap-FRAP technique requires a laser beam coupled with an epifluorescence microscope or, commonly, a confocal laser-scanning microscope, all of which are sophisticated and expensive instruments. To avoid photochemical and/or thermal cell injury, a highly sensitive light detector should be used; the minimization of illumination thereby reduces the amount of energy absorbed by the specimen. Excessive dye loading and/or fluence rate (fluence rate is identical to irradiance; that is, the flux density crossing the irradiated surface) may also cause cell damage or impairment of gap junctional communication by increasing the release of acids during intracellular hydrolysis of the ester, or of free radicals and reactive oxygen species during photobleaching. Cell-permeable dyes such as 2′, 7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Table 1) have been used in the gap-FRAP technique; however, as very strong laser light is required to rapidly photobleach fluorophores in a whole cell, the possible photodamage due to such intense laser illumination on cells needs to be assessed (40). Decreasing the bleach time, acquisition time, or the excitation beam intensity could avoid cell damage (56). In addition, the FRAP technique may be incompatible with multicolor imaging when other biochemical changes inside the cells need to be monitored by fluorescent sensors (40). In fact, an identical photobleaching protocol can lead to different photobleaching percentages as a function of cell type, resulting from either variability in optimal esterase activity or leakage of fluorescent dye by active transport (61). To apply Equation 1 to analyze dye transfer rates across gap junctions, it would be required that dyes diffuse much more rapidly in the cytosol than across Cx channels, so the rate-limiting step for intercellular dye passage is gap junction permeation. Whatever the gain in speed of the apparatus, the gap-FRAP method is not appropriate for measuring fast changes of the gap junction properties during application of a chemical or physical agent, as can be done by recording the electrical conductance (18).

Significant studies and applications. Gap-FRAP has been widely used for in vitro studies since 1986 (Table 2). As recently described by Zündorf et al.,

Table 2. Comparison of Methods for Studying Intercellular Communication

Properties	Micro-injection	Scrape Loading/ Electroporation	Gap-FRAP	Preloading Assay	LAMP	Radiolabeled Nucleotides Transfer	ICW	Dual Whole-cell Patch Clamp
Invasiveness	Invasive	Invasive	Noninvasive Possible photo-damage	Noninvasive	Noninvasive	Noninvasive	Invasive	Invasive
Cell integrity	Broken	Broken	Maintained	Maintained	Maintained	Maintained	Depends on stimulus intensity	Broken
Number of cells analyzed	Limited	High	Limited	Limited High (by FC analysis)	Limited	Limited	Limited	Limited to two cells
Temporal resolution	Low	Low	Good	Low	Good	Low	Good	High
Simplicity of set up	Time-consuming	Easy	Easy Requires high-power laser	Easy	Easy	Easy	Technically demanding and time-consuming	Technically demanding and time-consuming
Quantification of molecular transfer rate	Number of dye-coupled cells	Distance of diffusion from scrape line	Kinetic of dye recovery	Number of dye-coupled cells	Kinetic of dye diffusion	Autoradiographic labeling	Amplitude, velocity	Junctional conductance
Multicolor imaging	Feasible	Feasible	Not described	Feasible	Feasible	Feasible	Feasible	Feasible
Experiments	In vitro Ex vivo	In vitro Ex vivo (by scrape loading)	In vitro Ex vivo Potentially in vivo	In vitro	In vitro Potentially ex vivo and in vivo	In vitro	In vitro Ex vivo	In vitro
Main applications	Carcino-genesis Embryo-genesis Growth control Physiology	Carcinogenesis Embryogenesis Growth control	Carcinogenesis Embryogenesis Growth control	Carcinogenesis Embryogenesis Growth control Immunology (by FC analysis)	Metabolism	Metabolism	Physiology (e.g., astrocytes)	Physiology (cardiovas-cular system)

FC, flow cytometry; FRAP, fluorescence recovery after photobleaching; ICW, intercellular calcium waves; LAMP, local activation of molecular fluorescent probe.

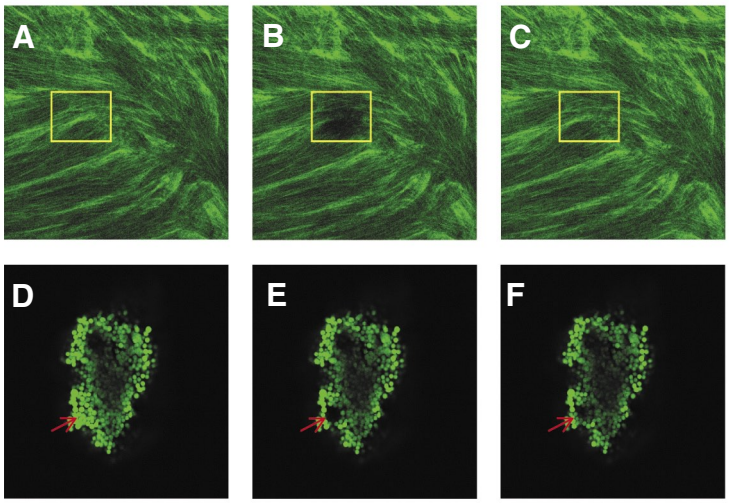

Figure 3. Representative images of dye transfer by gap-FRAP (fluorescence recovery after photobleaching) technique on multicellular three-dimensional model exposed to calcein acetoxymethyl ester (calcein-AM) as smooth muscle from mouse (A, B, C) and as head and neck squamous cell carcinoma spheroid (D, E, F). The bleached carboxyfluorescein molecules from a targeted area are indicated by a yellow rectangle or a red arrow, respectively. Each column introduces the three specific steps of the gap-FRAP technique: the left column shows recordings before photobleaching; the middle column, acquisitions just after the photobleaching time, and the right part shows fluorescence recoveries. A noticeable fluorescent gradient can be observed in the targeted area indicating functional gap junctions. Scale bars: 150 µm.

the gap-FRAP technique also permits detection of heterocellular dye transfer and analysis of the existence of functional heterocellular gap junctions between astrocytes and neurons in vitro (62).

Our group used the gap-FRAP technique to compare fluorescence recovery parameters after photobleaching in three cell types characterized by different patterns of phosphorylated Cx43, leading to different GJIC capacities being measured by two fluorescent tracers, 5(6)-carboxyfluorescein diacetate (CFDA) and calcein-AM (Table 1). We demonstrated that the choice of the dye did not significantly influence the fluorescence recovery percentages despite a cell line–dependent CFDA release, contrary to the fluorescence kinetic profiles. Calcein-AM seemed appropriate to allow discrimination in short duration experiments and to assess relative transfer constants not limited to one targeted cell but to a targeted surface in tissue analysis. However, the development of a sophisticated device to apply gap-FRAP on biopsies or in situ could be partly limited by the choice of the fluorescent dye. Due to its high spontaneous release, CFDA would not induce a clear delineation between the loading cells and the extracellular environment (60).

We have evaluated the potential of gap-FRAP to measure GJIC on a spheroid 3-D model with a confocal laser-scanning microscope as illustrated in Figure 3. In order to assess quantitative parameters characterizing spatial distribution of FRAP in three dimensions, the development of adapted behavioral models appears to be an essential condition. In fact, very few studies were reported in the literature concerning GJIC studies performed by gap-FRAP assays for a 3-D analysis from tissue organotypic slices (63,64), medial collateral ligament, and cartilage (59,65).

Preloading Assay or Parachute Assay: Incorporation of Membrane-permeable Fluorogenic Molecules

Principle. The preloading assay is a direct method developed by Goldberg et al. in 1995 to study functionality of GJIC (66). This method consists of preloading cells with a gap junction–permeable dye, such as calcein-AM, and then letting suspended loaded and unloaded cells form a confluent monolayer together. The principle of the parachute assay can be considered as similar, except that the loaded cells adhere to a monolayer of unloaded cells. As suspended loaded cells are added to the monolayer, the parachute assay requires formation of gap junction channels in order to measure subsequent intercellular communication. The parachute assay was described by Ziambaras et al. in 1998 to study whether stretch, an anabolic stimulus for osteoblasts, modulated direct intercellular communication in these cells (20). After a short time, the fluorescent dye passes from the "donor" cells to "acceptor" cells. Second, third, and higher order cells also take the dye, depending upon the degree of coupling (Figure 2E). Depending on the technique used, loaded cells make contact with unloaded cells within 15 min to 3 h, and the intercellular spreading of calcein can be observed and documented with, for instance, an epifluorescence microscope (20,66). The intercellular passage of calcein through gap junction channels results in the progressive increase of green fluorescent recipient cells (Figure 2E [c]). Under an epifluorescence microscope, intercellular communication is assayed as the number of unlabeled cells receiving fluorescent dye from a donor cell. This procedure is one way to visually quantify a large number of cells (66). Donor and recipient cells can also be assayed by flow cytometry (67). In this case, ester-loaded or unloaded cell populations can be labeled with a marker such as Di-I, which permanently labels cell membranes and makes cytometric identification of donor and recipient cells possible, even when both are of the same cell type (22).

Coupling time is defined as the time leading to the strongest coupling efficiency. Thus, the time course of the dye spreading depends not only on the intrinsic ability of recipient cells to allow the spreading of the dye, but also on the coupling of donor cells with recipient cells. Ultimately, the time course depends on the number of active gap junctions and on their permeability for the selected dye. In some experiments, carbenoxolone, a specific gap junction blocker, was added to ascertain whether the intercellular transfer assay was effectively dependent on GJIC (68).

Advantages. Using an AM ester, the parachute technique is a noninvasive method since integrity of the cells is maintained.

Flow cytometry enables simultaneous analysis of a large number of cells (at least 10^4 cells) (22). It ensures objectivity and statistically reliable data. Moreover, the accuracy and the high sensitivity to discriminate variation in fluorescence intensity have further advantages, especially when applied to measurements

of the effects of chemicals on junctional communications. The high sensitivity of the flow cytometry assay of dye transfer, too, allows a precise evaluation of the amount of transferred dye in recipient cells by analyzing their mean fluorescence intensity, using the appropriate detector (22). It resolves both the dynamics of initial cell-to-cell contact and the saturation state of coupling in monolayer cells (19). Finally, flow cytometry allows evaluation of multiple parameters at the same time (22). The quantitative end point, where the measurement of dye transfer concerns a large number of cells, gives a solid basis for a reliable result. The flow cytometer method may therefore be an interesting supplement to other dye transfer methods as a screening assay for substances with possible teratogenic and embryotoxic properties (17).

Limitations. This method is not applicable to the vast majority of adherent cell types in culture (31). By flow cytometry, nonspecific dye transfer of calcein has been observed in many studies (69,70). The preloading assay is also a time-consuming method that is influenced by the section studied (cells in different areas of the dish can behave differently), the resolution, and sensitivity of the microscope equipment (19). This technique cannot be used to investigate coupling within intact tissue.

Significant studies and applications. Flow cytometry is a useful approach to study GJIC of circulating cells (Table 2). To determine whether the changes in Cx43 expression produced by treatment with angiotensin II and ethanol are associated with functional changes, Bokkala et al. used the parachute assay to measure GJIC in white blood cells subjected to various treatment conditions (71). Assembly of Cx into gap junctions providing direct intercellular channels that link attached lymphocytes subpopulations (T, B, and natural killer lymphocytes) was demonstrated by using this dye transfer technique (72). By monitoring the calcein dye transfer from the stroma to leukemic cells, Paraguassu-Braga and colleagues described, using flow cytometry, the effect of GJIC on leukemic cell growth and death (73). Flow cytometry is widely used to study intercellular communication in different cell types, including endothelial cells, tumor cells, fibroblasts, and stromal cells (22,72,74,75) and enables comparison of dye transfer efficiency by different Cx isoforms (76).

LAMP (Local Activation of Molecular Fluorescent Probe)

Principle. Local activation of molecular fluorescent probe (LAMP) is an imaging assay of gap junctional communication based on a new generation of caged fluorophores (40). This noninvasive method is able to provide quantification of a permeable dye by multiple measurements. Photocaged fluorescent dyes have wide applications in tracking the spatiotemporal dynamics of molecular movements in biological systems. These caged tracers are weakly or nonfluorescent when key functional groups of fluorophores are masked by photolabile protecting groups (cages). Photoactivation removes the protecting group (uncaging) and abruptly switches on the fluorescence of parent dyes (Figure 2F).

A class of photoactivable fluorophores was developed and tested (40). An esterified nonfluorescent coumarin derivative can diffuse into cells, remaining nonfluorescent. The dyes are retained inside the cells due to their hydrolysis by cytoplasmic esterases, thus they can diffuse across cellular gap junctions. A brief illumination of a single cell or cell region efficiently photoconverts the trapped molecule into a fluorescent, gap junction–permeable species, and fluorescence is followed as a function of time in the coupled neighbor (77). Subsequent fluorescence imaging provides dynamic information of dye transfer from the donor cell to the receptor cell.

Advantages. Under ideal conditions, the technique is noninvasive, offering dynamic and quantitative information of dye transfer as well as multiple measurements of dye transfer in the same coupled cell pair to find changes of molecular permeability of Cx channels. The nonfluorescent 1-(2-nitrophenyl)ethyl (NPE)-caged coumarins/AM can be loaded into fully intact cells, and NPE-caged coumarins can be photolysed with a small dose of ultraviolet (UV) light. In contrast, techniques such as microinjection or patch clamping breach cell membranes and may cause loss or dilution of cytosolic factors involved in the gating of Cx channels. Maintaining cell membrane integrity is especially important for studying the regulation of gap junction communication by cellular biochemical changes.

The second advantage is that the LAMP assay can reliably detect changes in molecular permeability of Cx channels in coupled cell pairs. This crucial feature mainly results from two desirable properties of the newly developed caged fluorophore: the cell-loading efficiency of NPE-caged coumarins/AM and the small molecular size of caged coumarins. As NPE-caged coumarins/AM can be loaded into cells to fairly high concentrations, the LAMP technique can be applied several times in a coupled cell pair once the previous episodes of dye diffusion reach equilibrium. This allows multiple measurements of kinetics of dye transfer in cell pairs and thus detects changes in cell coupling. The small molecular size of caged coumarins ensures that the fluorophore can quickly diffuse across Cx channels to reach equilibrium in coupled cells. This rapid dye transfer facilitates quantitative analysis of kinetics of junctional diffusion using Fick's equation, and it improves the sensitivity of the detection when there are changes in the permeability of Cx channels. The last major advantage is that the LAMP assay can be used in conjunction with other fluorescent sensors for multicolor imaging. Because caged coumarins emit blue fluorescence, it spectrally complements a variety of fluorescence indicators that emit green or red light (40).

Applications. This technique is mainly applied to study the dynamic regulation of gap junction coupling by intracellular Ca^{2+} in individual cell pairs of human primary fibroblasts, for instance, and also to assess how Ca^{2+} influx affects junctional coupling on HeLa cells expressing Cx26 (77,78). LAMP is limited to examination of changes in coupling in individual cell pairs, although it is likely to be useful in multiwell plate assays with confluent cell monolayers as well. Furthermore, the combined use of the patch clamp method and LAMP would make it possible to correlate junctional conductance with permeability in cell pairs expressing different Cx, although cell volumes would still need to be taken into account (77).

ENDOGENOUS COMPOUNDS TRANSFER

Radiolabeled Nucleotides Transfer

Principle. Many molecules that are expected to be exchanged in vivo by

coupled cells have various dimensions and charges and hence may be restricted or facilitated in their passage through gap junction channels, depending on the type of Cx (12,31). Some investigators have examined the transfer of endogenous compounds by metabolic cooperation, fluorescent detection methods, or indirectly through induced metabolic effect (79–81). Major investigations have used the transfer of synthetic tracer molecules rather than endogenous metabolites. In the metabolic cooperation approach, a population of donor cells is incubated in the presence of an excess of a radiolabeled precursor (typically uridine) and then co-cultured with unlabeled recipient cells. Under such conditions, quantitative autoradiography enables evaluation of the transfer of the resulting metabolites from loaded to unloaded cells as a function of time. In this type of experiment, coupling is demonstrated by the autoradiographic labeling of the cytoplasm of recipient cells due to the incorporation in their ribonucleic acid of radiolabeled nucleotide synthesized within donor cells and transferred across Cx channels (31).

Advantages. Radioactive nucleotide transfer allows for a direct evaluation of the permeability of Cx channels to endogenous molecules. This technique is a highly sensitive method to appreciate transfer of molecules. Contrary to synthetic dyes, radioactive nucleotide transfer may accurately represent the ability of gap junction to mediate the transfer of biological endogenous metabolites (82).

Limitations. It is possible that incorporation by recipient cells of labeled nucleotides and nucleic acids lost by damaged donor cells may also be observed (31). Safety must be rigorously adhered to, particularly to deal with aerosol produced during cell sorting procedures, incubation, and metabolic labeling (83). While studies of metabolic cooperation can be used to garner information about transjunctional metabolites, they are not very quantitative, nor very specific, and are limited to the specific system they are used to investigate (4).

Significant studies and applications. Goldberg et al. developed a quantitative method to identify, by high-performance liquid chromatography screening, the entire set of native molecules that is exchanged between coupled cells using [^{14}C]-glucose as a radiolabeled precursor (84,85). Donor cells are labeled with a fluorescent marker larger than the gap junction channel limit to permit separation and, with radioisotope, to metabolically label transjunctional molecules. Goldberg et al. used this method to capture, identify, and quantify the junctional transfer of endogenous metabolites between C6 glioma cells transfected with Cx43 and Cx32 (84,86).

Intercellular Calcium Waves

Principle. Cytoplasmic calcium increases that propogate from cell to cell, which are called intercellular calcium waves (ICW), have been described in many different cell types by a number of investigators. This technique can measure gap junctional communication efficacy indirectly. Mechanical, chemical, or electrical stimulation of an individual cell initiates an increase in the intracellular Ca^{2+} concentration that spreads across the cell. At the cell border this intracellular Ca^{2+} wave is arrested but, after a brief delay, similar Ca^{2+} waves occur in adjacent cells. The repetition of this process results in the propagation of an intercellular Ca^{2+} wave through a limited number of cells (87). The distance the wave propagates appears to depend on the magnitude of the initial stimulus (88). The propagation of intercellular Ca^{2+} waves correlates with the presence of functional gap junctions and occurs in the absence of extracellular Ca^{2+} or following the microinjection of inositol triphosphate (IP_3) (87). Models for the propagation of ICW have been proposed (88,89).

In the technique, cells are loaded with a calcium-sensitive fluorescent dye. Two calcium indicators are mainly used, fura-2 (5-oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl) with two wavelengths of excitation (340 and 380 nm) and one of emission (90) or fluo-3 (glycine, N-[2-[[[[2-[bis(carb oxymethyl)amino]-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)phenox y]methyl]methyl]oxy]-4-methylphenyl]-N-(carboxymethyl), with one wavelength of excitation (490 nm) and one of emission (91). After loading, cells can be mechanically stimulated by manually or automatically moving a glass pipette onto the single-cell surface to transiently depress the apical membrane, or chemically stimulated by introducing an agent (92). Stimulation generates IP_3, which diffuses to neighboring cells through gap junction channels, and then triggers calcium release from IP_3-sensitive intracellular calcium stores (93). A variation in dye fluorescence intensity is measured in correlation with the propagation of ICW from cell to cell. Different parameters may be measured, such as velocity, amplitude, and efficacy of calcium wave spread (94).

Advantages. Fura-2 binding with calcium involves a shift of the excitation wavelength from 380 to 340 nm. The ratio of fluorescence signals measured during a response gives an accurate measure of ionic concentration independently of concentration of the fluorochrome or photobleaching caused by exposure of the whole field to the excitation light source (90). For fluo-3, one excitation wavelength leads to rapid kinetic measures. Fluo-3 can be used commonly with a confocal microscope associated with a 488 nm argon laser, which minimizes cell injury. Mechanical stimulation to induce ICW with a fine micropipette is an accurate protocol for single-cell stimulation (95).

Limitations. The mechanism whereby Ca^{2+} waves pass between cells is not completely elucidated: both intracellular and extracellular pathways were observed in some cell types under some conditions. Calcium waves may propagate from cell to cell by the secretion of a Ca^{2+} mobilizing agonist, potentially ATP, as a diffusible extracellular messenger (96,97). In fact, ICW depend not only upon the composition and distribution of gap junction channels but also the tissue expression of purinergic receptor subtype (96).

Measurements with fura-2 require equipment capable of fast switching between excitation filters or monochromators in order to perform both excitations successively. Acquisitions will be speed limited and, moreover, UV irradiation can induce cell injury. An accurate calibration of Ca^{2+} concentration is not convenient with fluo-3. Although both chemical and electrical stimulation trigger ICW, these methods have the disadvantage of simultaneously stimulating adjacent cells. Mechanical stimulation may induce microleaks in the plasma membrane and a reproducible stimulus strength is difficult to attain (95). A signaling cascade is

induced at the time ICW are stimulated; in addition to increased IP_3, other second messengers that may influence cellular response are produced (95).

Applications. Intercellular calcium waves were measured in a wide variety of primary cultured cells, including astrocytes (98,99), epithelial cells (100,101), hepatocytes (102,103), and endothelial cells (104). These observations have been extended to more integrated systems (105,106) and in perfused organs (107,108). A more sophisticated approach was proposed by Leybaert and Sanderson to stimulate single cells: the liberation of a cellular second messenger by flash photolysis of a caged compound to induce ICW (95). This method is based on the increase of cytoplasmic IP_3 by flash photolysis of caged IP_3 to activate the intracellular gap junctional pathway. They showed that flash photolysis of caged IP_3, and the resulting modulation of ICW, is a very sensitive technique to study cell-to-cell coupling. An exhaustive protocol was described by Leybaert and Sanderson's group in Reference 95.

CONDUCTANCE MEASUREMENT BY DUAL PATCH CLAMP

Principle. For the gap junction channels studied thus far, permeability and conductance are linearly related, so that measurement of conductance reliability reports the number of open gap junction channels (109). The first quantitative recordings of the single-channel current from an intercellular junction, presumably a gap junction, was made by Neyton et al., using the dual whole-cell patch clamp method in pairs of cells isolated from rat lacrimal gland (110). This sensitive method is of high temporal resolution for monitoring junctional conductance. The dual patch clamp technique is a powerful method for quantitative determination of junctional conductance (111). In the first applications of this method, each cell of a cell pair was impaled with two microelectrodes, allowing independent injection and voltage control. Currently, the most widely used dual-voltage clamp method is the double whole-cell voltage clamp technique with one suction pipette on each cell of a cell pair (109). Although at first, two-electrode voltage-clamp amplifiers were used (i.e., each cell had to be impaled by two microelectrodes), single-electrode voltage-clamp amplifiers have been used ever since the tight seal whole-cell recording technique became available (112).

The dual whole-cell patch clamp method is the most sensitive technique to detect functional gap junctions: a single gap junction channel can be recorded, in contrast to dye transfer. The method consists of separately controlling the membrane potential of each cell and measuring the corresponding currents. Figure 2G shows the equivalent circuit for a pair of cells.

Advantages. In contrast to dye transfer, evaluation of electrical conductance between cells is exquisitely sensitive. This is the most common method to assay the electrical properties of gap junctions. The technique has been used to study ionic permeability and selectivity of Cx channels. Measurements of electrical conductance between cells can be used to determine the number of gap junction channels that are open within a given period of time (4).

Limitations. Intercellular current measurements do not necessarily correlate with the kinetics of molecular transfer through gap junctions (113), and quantification of junctional conductance can be especially problematic in well-coupled cells where contributions of series resistance can outweigh junctional resistance (109). Permeability of the gap junctions is a measure of the cell-to-cell exchange of molecules driven by their kinetic energy and should not be confused with the electrical conductance that describes the ionic currents driven by a gradient of electrical potential. The information acquired by these two kinds of data are therefore not equivalent in the sense that knowledge of the conductance of a unit junctional channel for a particular ion cannot be used to predict the permeability of a given molecule (18).

In addition, analysis of electrical conductance is a slow, labor-intensive, and expensive technique. Measurement of junctional conductance (G_j) with patch clamp amplifiers can, due to series resistance problems, be subject to considerable errors when large currents are measured. Series resistance is a problem mainly in situations in which gap junction resistance (R_j) and/or membrane resistance is low, because large currents will flow (Figure 2G), and, according to Ohm's law, this will result in large voltage drops across the series resistance. In those situations, errors in the measurement of G_j can become as large as 70%. To correct for the errors introduced by series resistance, several correction formulas were proposed that take into account either series resistance alone or a combination of series resistance and cell input resistance (114). All these formulas depend critically on an exact estimate of the series resistance, which is not always easy to obtain (112). Under conditions of low membrane resistance, it is necessary to use a correction method that takes both membrane and series resistance into account (31). Thus, dual patch clamp recording is a quantitative technique to determine static and dynamic electrical characteristics of gap junction channels; however, as with most experimental techniques, the dual patch clamp has several drawbacks, which vary from quantitative errors in measured conductance or underestimation of voltage dependence to unwanted perfusion of the cytoplasm (111). The pipette solution is brought into contact with the cytoplasm by rupturing with gentle suction, the membrane under the tip of the pipette. The small tip becomes partially clogged with an access resistance to the cell interior that is considerably larger than resistance of the pipette alone. The pipette series resistance has been reported to increase during an experiment, probably due to an increase in pipette clogging or a slight drift of the cell surface away from the pipette (109).

Significant studies. As cell dissociation and culture procedures have been improved, the dual whole-cell recording technique has been applied to a wide variety of tissue (115). Dürig et al. showed that functional Cx43-type gap junctions exist between stromal cells and immature hematopoietic progenitor cells and may provide an important regulatory pathway in hematopoiesis (116). The authors used electrophysiological methods to assess cell-to-cell communication that have been shown to be superior to dye-coupling experiments in terms of sensitivity and their applicability to different gap junction subtypes. Junctional conductance measurements can be used to quantitate and compare the transfer of specific molecules through gap junctions formed by one type of Cx; moreover, substituting ions in the patch clamp electrodes can reveal the preferential transfer of specific ions that travel through different gap junction isoforms (4,117–119). The size

permeability for small molecules and the connected electrophysiological properties of a gap junction are dependent on the gap junction pore size as generated by the respective Cx isoforms (120). In experiments described by Bukauskas et al., communication-incompetent cell lines transfected with Cx43 fused to enhanced green fluorescent protein were examined in order to compare Cx43 distribution determined by fluorescence microscopy and electrical coupling measured at single-channel resolution in living cell pairs (121). Dual whole-cell patch recording was combined with imaging microscopy to show that only gap junction channels assembled into junctional plaque are functional.

CONCLUSION

Here, we have reviewed several techniques allowing the characterization of GJIC. These methods are not equivalent but provide complementary information (Table 2). Dye transfer methods have been considered to be the reference technique for 40 years. LAMP, the more recent imaging method, based on a newly described photoactivatable fluorophore, promises to illuminate how gap junctions mediate intercellular coupling. The scrape-loading technique and microinjection can be effectively used as tools to determine the qualitative presence or absence of GJIC. Quantitative methods such as gap-FRAP and LAMP facilitate measurement of kinetics through gap junction channels to determine, for example, the permeability constant. Conductance measurement by dual patch clamp is considered the most sensitive method to precisely measure and follow modifications in GJIC; nevertheless, in order to appreciate the transfer of endogenous molecules, the transfer of radioactive nucleotides appears to be a sensitive method.

The methods described in this review are mainly used for in vitro applications. Some of them have been extended successfully to ex vivo studies (Table 2). Dye transfer by microinjection, scrape loading, and gap-FRAP can be used today for ex vivo measurements, and the LAMP technique seems appropriate to perform future 3-D studies.

In the coming years, the major challenge for the intercellular communication field will be to further investigate the functionality of gap junction channels in situ, under both physiological and pathological conditions.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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